Detection of Human Metapneumovirus Antigens in Nasopharyngeal Secretions by an Immunofluorescent-Antibody Test

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Received 7 September 2004/Returned for modification 14 October 2004/Accepted 20 October 2004

Human metapneumovirus (hMPV) is a recently discovered pathogen associated with respiratory tract infections, primarily in young children, immunocompromised individuals, and elderly individuals. Reverse transcription-PCR (RT-PCR) has been reported to be a more sensitive method for the diagnosis of hMPV infections than virus isolation by culture and serological study. However, there has been no report on rapid methods, such as an immunofluorescent-antibody test or an enzyme-linked immunosorbent assay, for the detection of hMPV antigens in nasopharyngeal secretions. In this study, we compared an indirect immunofluorescent-antibody test (IFA) with a monoclonal antibody with RT-PCR for detection of hMPV in nasal secretions from 48 hospitalized children with respiratory tract infections. Fifteen of the 48 children were positive for hMPV by RT-PCR. IFA results were positive for 11 of the 15 RT-PCR-positive children (sensitivity, 73.3%) and 1 of the 33 RT-PCR-negative children (specificity, 97.0%). Although the sensitivity of IFA is lower than that of RT-PCR, IFA is a rapid and useful test for the diagnosis of hMPV infections in children.

Human metapneumovirus (hMPV), a novel respiratory virus, has recently been recognized as an etiologic agent of respiratory tract infections in children and adults (2, 23, 25). Since hMPV is genetically related to human respiratory syncytial virus (hRSV), hMPV and hRSV are grouped in the same subfamily, subfamily *Pneumovirinae*, of the family *Paramyxoviridae* (23). The clinical manifestations and epidemiologies of hMPV and hRSV have been reported to be very similar (3, 21, 24, 27). Both viruses are separated into two groups by genetic differences (12, 14, 23). The seasonality of hMPV infections resembles that of hRSV infections, and the epidemic season of hMPV is from winter to early spring (6, 25). Serological studies have shown that all children are exposed to hMPV by the age of 5 to 10 years (5, 23). Repeated infections with hMPV occur throughout life, even in early childhood (2, 6, 7, 14). Previous studies have indicated that hMPV causes mild respiratory tract infections in healthy adults (2, 22, 25). However, it has been shown that children under 2 years of age, elderly people over 50 years old, and immunocompromised patients are at greater risk of lower respiratory tract infections, such as bronchitis, pneumonia, and bronchiolitis (25). hMPV has been revealed to be associated with acute wheezing in children (6, 13, 25, 29).

Four principal methods are used for the diagnosis of respiratory virus infections: virus isolation by culture, antigen detection, RNA or DNA detection, and serological study. In previous studies (6, 15, 17), virus isolation by culture, reverse transcription-PCR (RT-PCR), and serological study have been used for the diagnosis of hMPV infections. Virus isolation by culture with tertiary monkey (*Macaca mulatta*) kidney cells, LLC-MK2 cells, or Vero cells is specific and a “gold standard” method (23, 25). However, we could not isolate hMPV by culture from two-thirds of RT-PCR-positive samples (6). Se-

rological study is retrospectively important for differentiation between primary infection and reinfection with hMPV. However, the immunoglobulin G (IgG) and IgM antibody responses to hMPV in the acute phase are not useful for the diagnosis of hMPV infections (6). Therefore, RT-PCR is concluded to be the most sensitive and specific procedure (6, 25). However, RT-PCR can be performed only in special laboratories, and it takes more than 6 h to obtain results.

Two rapid antigen detection methods are available: an immunofluorescent-antibody test and an enzyme-linked immunosorbent assay (ELISA). The ELISA method with mouse polyclonal antibodies to hMPV has been reported to enable detection of hMPV antigens of hMPV-infected cells in culture (28). However, there has been no study on the detection of hMPV antigens in clinical samples. The immunofluorescent-antibody test of respiratory epithelial cells has been shown to be a very useful method to detect other respiratory viruses, such as hRSV, parainfluenza virus, adenovirus, and influenza virus (11). The purpose of this study was to compare the rate of virus detection in nasopharyngeal secretions by an indirect immunofluorescent-antibody test (IFA) with that by RT-PCR.

MATERIALS AND METHODS

Patients and nasopharyngeal swab samples. From April to May 2004 we collected nasopharyngeal swab samples from 48 children who were admitted to Hokkaido Social Insurance Hospital, Kohnan Hospital, Kosei General Hospital, and Tenshi Hospital in Sapporo, Japan, due to respiratory symptoms. Two nasopharyngeal swab samples were collected from each patient. One sample was placed in 2 ml of Eagle's minimum essential medium for IFA, and the other was placed in 500 μ l of RNAzol B (Tel-Test, Inc., Friendswood, Tex.) for RT-PCR. The samples were collected within 7 days after the onset of illness, after the possibility of infection with hRSV or influenza A or B virus was excluded by a rapid antigen detection test. The male-to-female ratio was 1.5 to 1. The mean age of the 48 children was 1.5 years (age range, 1 month to 4.9 years). The final diagnoses for the children are shown in Table 1. All samples were collected after informed consent was obtained from the children's parents. As negative controls, we collected five samples from healthy adults and three samples from children with infected with hRSV, as confirmed by rapid antigen detection tests.

IFA. Nasopharyngeal swab samples for IFA were gently mixed to create a free cell suspension. The cell suspension was centrifuged, and the cell pellet was

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TABLE 1. Detection of hMPV by RT-PCR and IFA

Diagnosis	No. of samples that were:				Total no. of samples
	RT-PCR and IFA positive	RT-PCR positive and IFA negative	RT-PCT negative and IFA positive	RT-PCR and IFA negative	
Wheezy bronchitis	6	0	0	15	21
Bronchitis	3	2	0	3	8
Pneumonia	1	2	1	5	9
Laryngotracheitis	1	0	0	0	1
URTI ^a	0	0	0	8	8
Bronchiolitis	0	0	0	1	1
Total	11	4	1	32	48

^a URTI, upper respiratory tract infection.

resuspended in 50 to 400 µl of phosphate-buffered saline (PBS). Cell smears were prepared by spotting 1 drop of the cell suspension onto a slide glass. Then, the smears were air dried and fixed in acetone for 10 min. For detection of hMPV antigens by IFA, we used an anti-hMPV mouse monoclonal IgG antibody (monoclonal antibody 1B7) from CHEMICON International, Inc. (Temecula, Calif.), which recognizes hMPV antigens of both groups A and B and of both subgroups 1 and 2. Before the IFA with the monoclonal antibody was started, we took hMPV-infected LLC-MK2 cells for use as hMPV-positive cells for IFA and reacted them with the monoclonal antibody and also with hMPV-positive human serum. Two patterns of IFA staining, granular and diffuse, of the hMPV-infected

LLC-MK2 cells with the monoclonal antibody are shown in Fig. 1A and B, respectively. There was no difference between the frequency of hMPV antigen-positive cells obtained with the monoclonal antibody and that obtained with hMPV-positive human serum. Smears were incubated for 30 min at 37°C with the anti-hMPV mouse monoclonal antibody at a dilution of 1:80 and nonimmune mouse antibody as a negative control. They were then incubated for 30 min at 37°C with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) at a dilution of 1:40 with 0.001% Evans blue. After incubation, they were washed twice in PBS for 10 min each time, air dried, and mounted with PBS-glycerin (1:1). We confirmed that there were at least more

FIG. 1. Typical IFA staining of hMPV-infected LLC-MK2 cells (A, granular staining [white arrow]; B, diffuse staining) and nasopharyngeal epithelial cells (C, IFA-positive staining; D, IFA-negative staining) with the anti-hMPV monoclonal antibody (monoclonal antibody 1B7).

FIG. 2. Phylogenetic analysis of hMPV fusion nucleotide sequences. A tree was constructed by the neighbor-joining method with 100 bootstraps with random sequence addition. The length of the horizontal line represents the number of substitutions per site. Bootstrap values over 70% are shown. Avian pneumovirus subgroup C (APV; GenBank accession number AF187152) was the outgroup and was used to root this tree. *, samples negative for hMPV by IFA. The hMPV sequence analysis included the following isolates (GenBank accession numbers are given in parentheses): isolates 94-1 (AF371342), 99-1 (AF371344), 00-1 (AF371337), and 93-1 (AF371341) from The Netherlands (23) and isolates JPS04-396, JPS04-407, JPS04-389, JPS04-372, JPS04-379, JPS04-394, JPS04-399, JPS04-402, JPS04-405, JPS04-395, JPS04-401, JPS04-409, JPS04-406, JPS04-411, and JPS04-417 (AY653162 to AY653176) from Sapporo.

than 100 epithelial cells on one spot at $\times 400$ magnification under a fluorescent microscope. We examined approximately 100 to 500 epithelial cells for the presence of specific fluorescence patterns, such as granular staining and diffuse staining, in the cytoplasm. Typical IFA-positive and IFA-negative staining patterns of nasopharyngeal epithelial cells are shown in Fig. 1C and D, respectively. The presence of at least one cell with a specific staining pattern was defined as a positive result.

RT-PCR method and sequencing. The RT-PCR method used in this study was described previously (6). Briefly, cDNA was synthesized from total RNA extracted from each nasopharyngeal swab sample. A forward primer with a sequence of 5'-CTGTTCCATTGGCAGCAATA-3' and a reverse primer with a sequence of 5'-TCAAAGCTGCTTGACACTGG-3' were used to detect the fusion protein gene of group A. A forward primer with a sequence of 5'-AATCGGGTTGAATCATCAA-3' and a reverse primer with a sequence of 5'-GCTGTTACCTTCAACTTTGC-3' were used to detect the fusion protein gene of group B. Sequencing of the 80 nucleotide bases of the PCR product was performed by using a BigDye Terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Foster City, Calif.) with an ABI Prism 310 genetic analyzer (Perkin-Elmer Applied Biosystems). The sequences of the 15 hMPV strains were confirmed by sequence analysis (Fig. 2). Phylogenetic trees were constructed with CLUSTAL W software and the Phylip (version 3.6; alfa3) program (DNADist and Neighbor software).

Nucleotide sequence accession numbers. The sequences of the 15 hMPV strains have been deposited in GenBank under accession numbers AY653162 to AY653176.

RESULTS

The results for 48 children tested by RT-PCR and IFA are shown in Table 1. All of the children positive for hMPV by either RT-PCR or IFA had lower respiratory tract infections, including wheezy bronchitis, bronchitis, pneumonia, and laryngotracheitis. Fifteen of the 48 children were positive for hMPV by RT-PCR. IFA results were positive for 11 of the 15 RT-PCR-positive children and for 1 of the 33 RT-PCR-negative children. Approximately 0.5 to 2.0% of the cells had specific staining. No specific staining was observed in samples from the healthy adults or the hRSV-infected children. The sensitivity and specificity of IFA were 73.3 and 97.0%, respectively. The rate of agreement of the results between IFA and RT-PCR was 89.6%.

A phylogenetic tree constructed on the basis of the hMPV fusion nucleotide sequences of the 15 hMPV strains is shown in Fig. 2. Positive IFA results were obtained with the hMPV strains of both groups A and B and subgroups 1 and 2, confirming the reactivity of the monoclonal antibody. For the 15 RT-PCR-positive children, the mean ages of the 11 IFA-positive children and the 4 IFA-negative children were 1.2 ± 1.3 years (standard deviation) and 1.8 ± 1.0 years (standard deviation), respectively. There was no significant difference between the mean ages of the two groups ($P = 0.44$ by Student's t test).

DISCUSSION

We applied IFA to the detection of hMPV antigens in nasopharyngeal secretions for the rapid diagnosis of hMPV infection. The present study showed that IFA with an anti-hMPV mouse monoclonal antibody could detect hMPV antigens in nasopharyngeal secretions with 73.3% sensitivity and 97.0% specificity compared with the results of RT-PCR. We have reported that RT-PCR is a more sensitive method than serological studies and culture for the diagnosis of hMPV infection (6). In a previous study, hMPV was not detected by culture from two-thirds of RT-PCR-positive children. Although we could not perform virus isolation by culture in this study, IFA seemed to be more sensitive than virus isolation by culture.

Four RT-PCR-positive and IFA-negative cases were considered false negative. The false-negative results obtained by IFA might have been caused by technical problems, such as the use of unsuitable smears, and by the sampling period. In general, IFA is less sensitive than RT-PCR for the detection of viruses (4, 10, 19, 20). Furthermore, the sensitivities of various methods for the detection of respiratory viruses decrease in the convalescent phase of illness (6, 8, 9, 26). In fact, additional samples obtained from three RT-PCR-positive children during a period of 8 to 14 days after the onset of illness were RT-PCR positive and IFA negative (data not shown). On the other hand, only one child was RT-PCR negative and IFA positive in the present study. This might have been due to the classification of a misleading nonspecific fluorescence as specific staining.

IFA and ELISA are two major rapid tests for the detection

of virus antigens in nasopharyngeal secretions. In this study, we found that IFA is useful for the diagnosis of hMPV infections. In general, the detection of viral antigens by IFA and ELISA requires sufficient amounts of viral antigens in the clinical samples, including nasopharyngeal secretions. Experimental hMPV infection in cynomolgus macaques has shown that virus antigens are detectable mainly in ciliated epithelial cells throughout the respiratory tract, such as the nasal septum, trachea, and bronchus, and that macaques shed virus in nasopharyngeal secretions at high titers (15). In this study, hMPV antigens were detected in the epithelial cells of the postnasal cavity by IFA, indicating that nasopharyngeal secretions contain sufficient hMPV antigens to be detectable by other immunological detection methods such as ELISA.

IFA and ELISA each has merits and demerits (18). ELISA is easy to perform and provides results more objective than those provided by IFA. Therefore, many antigen detection ELISAs are used as rapid procedures for the diagnosis of respiratory tract infections in clinics. Although IFA requires some special techniques, it enables confirmation that each nasopharyngeal sample contains certain numbers of epithelial cells under a fluorescent microscope. Furthermore, there have been many reports that IFA is more sensitive than ELISA for the detection of respiratory virus antigens (1, 16, 17).

In this study, we showed that IFA is a useful and rapid test for the diagnosis of hMPV infections. However, the development of tests that are easier to perform in clinics, such as ELISA, is necessary for the appropriate treatment of patients.

ACKNOWLEDGMENTS

This study was partially supported by a grant-in-aid for exploratory research (grant 14657179 [2002]) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a grant-in-aid for the 21st Century Center of Excellence for Zoonosis Control from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Nasopharyngeal swab samples were kindly provided by Hiroyuki Sawada, Tsuguyo Nakayama, Yoshiko Taten, Yoshikazu Kinugawa, and Yasuto Suzuki of Hokkaido Social Insurance Hospital; Yutaka Takahashi, Takaaki Shikano, and Fumie Fujiwara of Kohnan Hospital; Mutsuko Konno, Michiko Takahashi, Kouhei Sato, and Emi Mino of Sapporo Kosei General Hospital; and Koga Yasutsugu, Makoto Kaneda, and Shohei Konishi of Tenshi Hospital. We also thank Kunihiro Kobayashi, emeritus professor of Hokkaido University, for giving us suggestions and Stewart Chisholm for proofreading the manuscript.

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